NR-201-575
CON441

(2)

DEFENSE ADVANCED RESEARCH PROJECTS AGENCY

R & D STATUS REPORT

ARPA ORDER NO.
#4507

PROGRAM CODE NO.
#N00019

CONTRACT NO.
N00014-82-K-0680

CONTRACT AMOUNT
$438,905.00

SHORT TITLE OF WORK

ULTRASENSITIVE DETECTION OF CHEMICAL SUBSTANCES

REPORTING PERIOD
JULY 1, 1982 - SEPTEMBER 24, 1982

EFFECTIVE DATE OF THE CONTRACT
JULY 1, 1982

EXPIRATION DATE OF THE CONTRACT
JUNE 30, 1983

PRINCIPAL INVESTIGATOR
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I. PROGRESS REPORT FOR PERIOD OF JULY 1, 1982 TO SEPTEMBER 24, 1982

The above contract was officially signed on August 17, 1982 retroactive to July 1, 1982. Permission to purchase major equipment was received from the Office of Naval Research August 20, 1982. As of the date of this report most major equipment has been ordered and is in the laboratory or in shipment. The only remaining items of equipment to be ordered are: a laminar flow hood, a Ludlum survey meter and dosimeter, a gamma counter, and a fluorescent microscope. These items will be ordered as the need arises.

ACETYLCHOLINESTERASE (AChE) ASSAY

Almost all procedures and assays to be developed during the course of this contract require an assay procedure that will quantitate the presence of acetylcholinesterase. For the determination of AChE in stock samples, serum and any preparation of AChe in solution we are using the colorimeter assay of Ellman (1). This procedure is outlined briefly below:

ASSAY PROCEDURE

REAGENTS

1. Phosphate buffer - 0.1 M pH = 8.0.
2. Acetylthiocholine - 0.75 M (21.67 mg/ml) in phosphate buffer pH = 8.0.
3. Dithiobisnitrobenzoic acid (DTNBA) 0.1 M (39.6 mg/10 ml phosphate buffer pH = 7.0 + 15 mg sodium bicarbonate).
4. Acetylcholinesterase - 5 u/ml H_2O - dilute preparation from sigma 1 - 100.

REACTION

Cuvettes	Phosphate Buffer	A-CH	DTNB	AChE
1	3.050 ml	20 ul	100 ul	-
2	3.050 ml	20 ul	100 ul	50 ul

Absorbance read at 405 nm.

Figures 1 and 2 show a Standard Curve and Kinetic Data for this AChE Assay. This assay can detect as little as 10^{-2} units (10ngm) of AChE. With prolonged incubation (2 hours) we can detect and quantitate less than 10^{-3} units (sub-ngm) amounts of AChE. This sensitivity is more than adequate for any estimation of AChE used in the development of the ultrasensitive assay.

The colorimeter assay is, also, versatile. Because the optical density is read at 405 nm the procedure is directly adaptable to measurement by Elisa reader at 405 nm which is the same wavelength used for peroxidase measurements. We have modified the assay to measure the amount (activity) of AChE bound to the solid surface of microliter plate wells.

PLATE ASSAY PROCEDURES:

Each well receives the following proportions of reagents in the order stated:

1. 150 ul of 0.1 M phosphate buffer pH = 8.0.

2. 5 ul of 0.75 M acetylcholine iodide.

3. 5 ul of 0.019 M dithiobisnitrobenzoic acid.

Solutions are read at 405 in Micro Elisa after 10 minutes of incubation at room temperature. The optical path length is approximately 0.473 cm.

As will be shown in the following section this assay is very sensitive and is capable of detecting less than 10^{-3} units of AChE. This assay is currently used to determine AChE binding to a variety of plastic supports under different conditions of binding. This test is, also, used to test the stability of bound AChE with time.

BINDING OF AChE TO SOLID SUPPORTS (MICROLITER PLATE WELLS)

AChE coated microliter plates are necessary for both monoclonal antibody selection procedures, and for formulation of the ultrasensitive assay. Most of our initial experiments have been directed toward passive binding of the AChE protein to plastic wells. The passive procedures are relatively simple, unlikely to interfere greatly with the activity of the bound protein and adequate for monoclonal antibody selection procedures. The basic procedure for binding AChE to polyvinyl and polystyrene wells is outlined below:

1. Dilute AChE with PBS at pH = 7.4 for polyvinyl plates and in carbonate buffer at pH = 9.46 for polystyrene plates.

2. Add 100 ul of each enzyme dilution to individual wells.

3. Let it incubate overnight at 4° in moisture box.

4. Discard supernatant and wash each well 3x with PBS.

Our results with passive binding are summarized in Table 1.

From Table 1 it appears that polystyrene binds more fully than polyvinyl and the highest efficiency of binding is found at lower concentrations. However, binding to both polyvinyl and polystyrene plates is adequate for purposes of monoclonal antibody selection. One problem remains. We find that AChE bound to a polyvinyl does not appear to be stable and the activity upon dry storage at 4°c is lost within a week. We have not completed experiments with this problem, however, at this time we feel that a solution can be found and the nature of this problem does not stop progress in the selection procedures for monoclonal antibodies.

RABBIT ANTISERA

Rabbit antisera to AChE are necessary as a control in the monoclonal selection procedure. Two procedures have been used to raise anti-AChE antibody in rabbits (2,3). Our procedure is based on elements from both of these cited methods. Two New Zealand white rabbits were each inoculated (8/30/82) with 0.5 ml of antigen containing 1 mgm AChE sigma type VI and a 1:2 dilution of Freunds complete adjuvant.

Injection was accomplished through the foot pads. A serum sample was taken (9/1/82) and stored for later assay. A second injection was administered (9/20/82) using the same conditions as above; except, that incomplete adjuvant was used and injection was done subcutaneously. Serum will be taken after a third injection and purified for monoclonal antibody selection procedures.

FLUORESCENT SIGNAL PACKETS

Fluorescent beads (covashperes) were purchased from the Covalent Technology Corporation these beads vary in size from 0.5 um to about 1 u. They are hydrophilic with low nonspecific adsorption characteristics. These beads can be counted with a 10 power objective using the Ziess Universal Microscope with epi-fluorescence, however, a 16 or 25 power objective would improve counting performances.

Initial experiments tested nonspecific binding between uncoated beads and polyvinyl and polystyrene surfaces. About 10^6 beads were added to each well and allowed to react at room temperature for 1 hour then washed out with PBS or PBS tween 20. After washing and sonication approximately 6×10^2 beads/cm^2 were found. This number was unaffected by washing procedure.

Coating both beads and wells with calf serum reduced nonspecific binding below countable levels. This was especially true in the polyvinyl plates. Very shortly we will improve our counting ability when we receive a stage and net micrometer for the microscope.

II. CHANGE IN KEY PERSONNEL

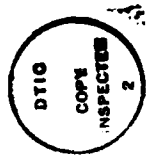
1. Josephine Morris, Research Technician IV.

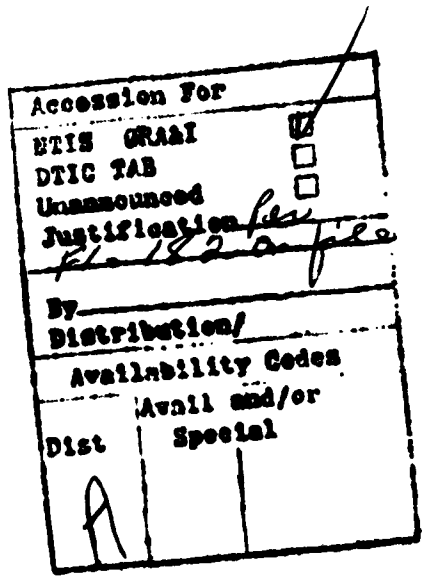
2. Susan Gray, Research Technician II.

3. Jeanette Rampone-Gulder, Staff Assistant.

III. FISCAL STATUS

See first quarterly report.





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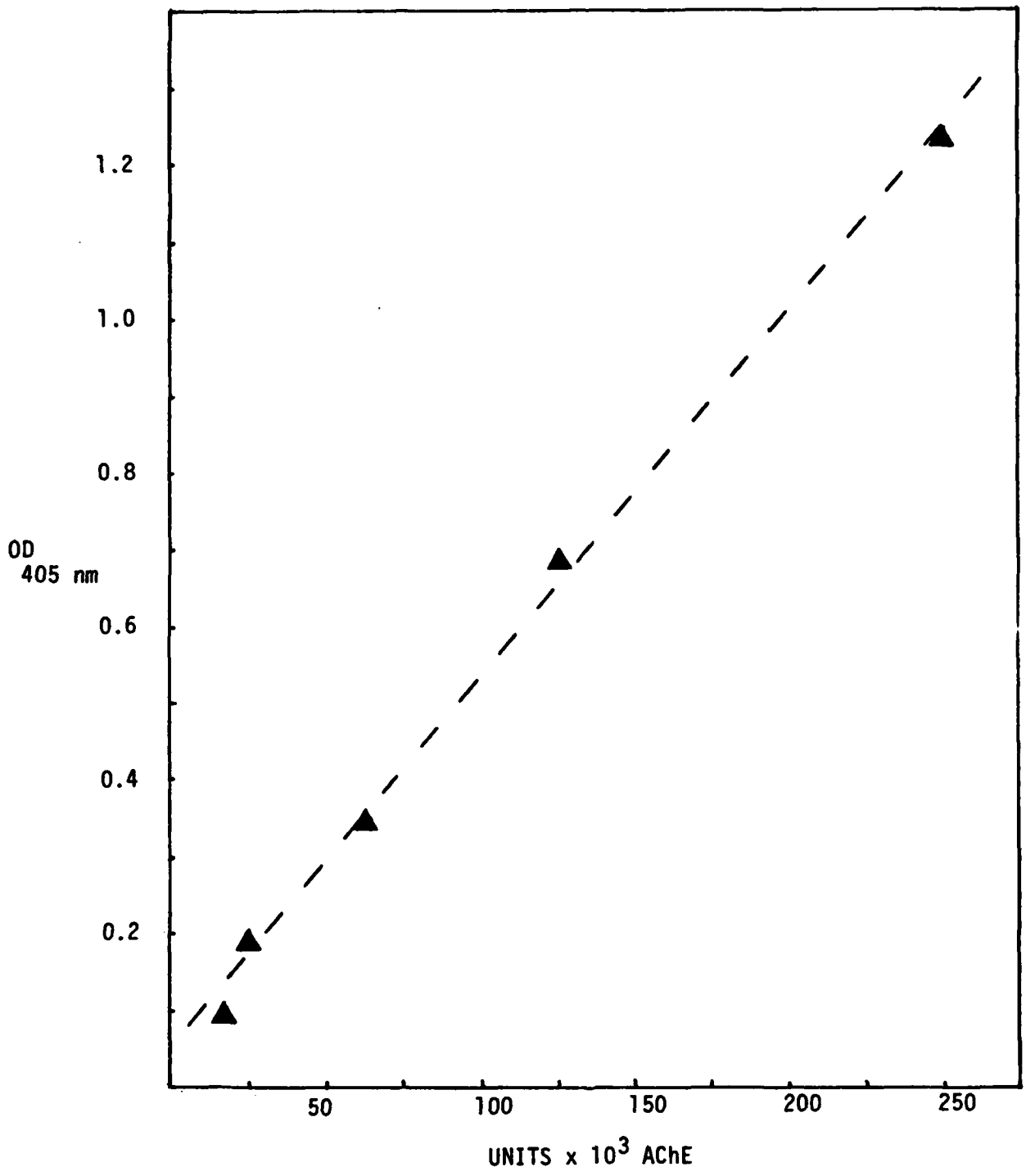

Figure 1 - STANDARD CURVE

Incubation time was 20 minutes.

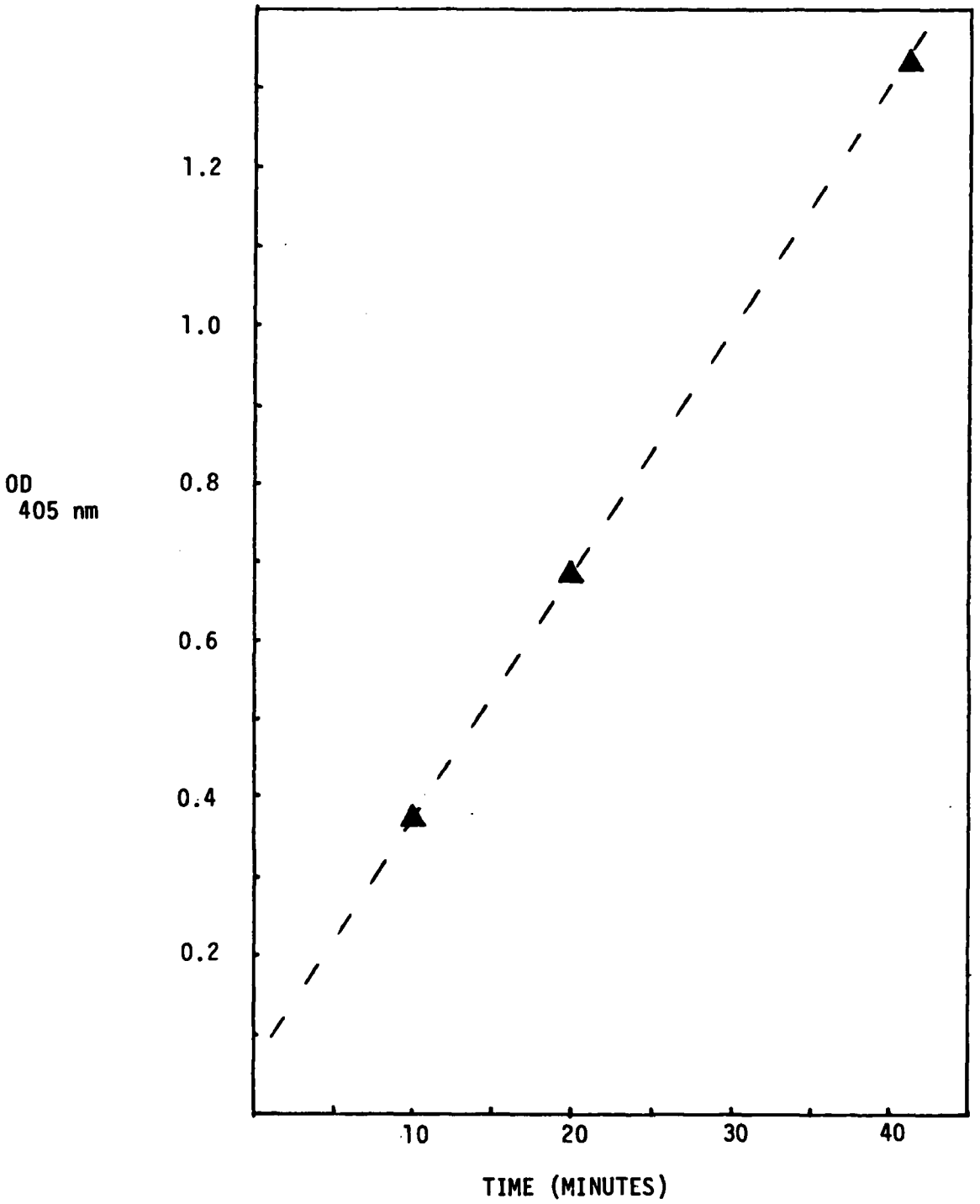

Figure 2 - AChE KINETICS

AChE concentration was 0.125 units

POLYSTYRENE PLATES

UNITS USED	OD 405	BOUND UNITS	% BOUND
.0125	.795	1.98×10^{-3}	15.8
.025	.923	2.29×10^{-3}	9.2
.05	1.099	2.73×10^{-3}	0.55
1.0	1.232	3.06×10^{-3}	0.31
2.0	1.333	3.31×10^{-3}	0.17
	POLYVINYL PLATES		
.0125	.008	0.020×10^{-3}	0.16
.025	.125	0.38×10^{-3}	1.52
.50	.646	1.6×10^{-3}	0.32
1.0	.801	2.0×10^{-3}	0.20
2.0	1.124	2.8×10^{-3}	0.14

Table 1 - PLATE COATING